Zn-Finger Transcription Factor Genes as Regulatory Targets of *Drosophila* Prospero and Cyclin E During Neurogenesis

A Senior Honors Thesis presented for
Graduation for Research Distinction
By

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INTRODUCTION

Neurogenesis within Drosophila begins with the separation of neural progenitor cells (neuroblasts) form the ventral neuroectoderm and the procephalic neuroectoderm. To do this the neuroblasts must move inward to the interior of the ectoderm so that they may build up the primordium of the central nervous system (Campos-Ortega, 1993). The other cells of the neuroectoderm take on a different developmental state as they develop as epidermoblasts. The determination of whether cells follow the neural pathway to form the central nervous system or the peripheral nervous system (PNS) requires cell to cell communication (Hartenstein, 1984). This communication depends on the function neurogenic genes and proneural genes. This model for cell determination by cell to cell communication is substantiated by the function of the AS-C genes in neural determination and the effects of E(spl)-C in epidermogenesis. These and other neurogenic and proneural genes are regulated by certain transcription factor genes.

The development of the Drosophila nervous system requires the regulatory functions of numerous transcriptional regulators. Drosophila Prospero (Pros) is a well studied transcription factor regulator whose function is crucial for normal nervous system development in Drosophila (Vaessin et al., 1991). Pros was found in the Vaessin laboratory to coordinate the developmental decision between cell cycle and neuronal differentiation (Li and Vaessin, 2000). Specifically, it was shown to regulate the mitotic activity and transcriptional expression of a range of target genes. More recently, the Vaessin laboratory showed that Pros cooperatively interacts with Cyclin E (CycE) in the regulation of various target genes, such as *nerfin-1*, during Drosophila neurogenesis (Vaessin, unpublished observation). While the dynamics of Pros dependent *nerfin-1* (a

zinc nervous finger transcription factor encoding gene) expression have been researched and determined by *in situ* hybridization of Drosophila embryonic tissues, other zinc-finger transcription factors who may represent Pros regulatory targets, have yet to be well studied. *nerfin-1* regulates the development of Drosophila central nervous system at several levels, including cell fate and early axon guidance decisions. (Kuzin et al., 2005). Several additional Zn-finger transcription factor genes have been identified in the Vaessin laboratory by microarray analysis as potential regulatory targets of Pros and CycE. Zinc finger transcription factor genes are genes that code for proteins which bind to a specific segment of DNA and control the transcription of that segment of DNA into mRNA (Ashraf, 2004). They gain the zinc finger part of their name because they coordinate one or more zinc ions to help the protein fold in a certain way.

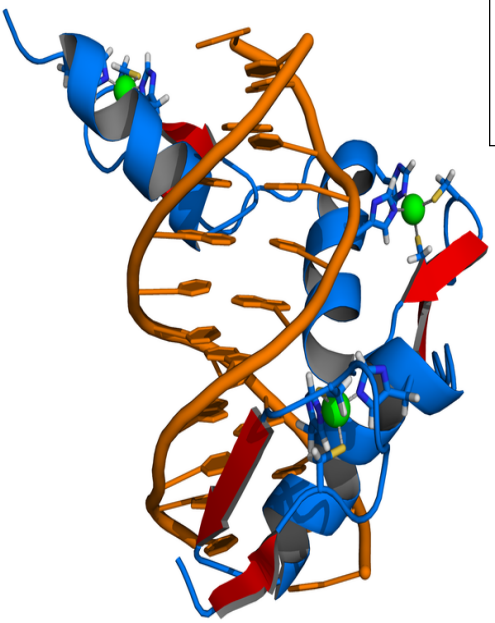
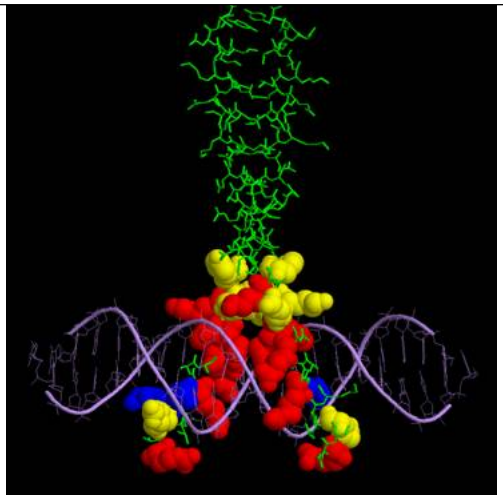


Figure 1: (left) Representation of the protein Zif268 (blue) containing three zinc fingers in complex with DNA (orange). The coordinating amino acid residues and zinc ions (green) are highlighted.
Narlikar GJ, Fan HY, Kingston RE (February 2002)

Figure 2:
(below) Representation of the binding of zinc finger transcription factor protein with a DNA double helix. Will either activate (upregulate) or suppress (downregulate) DNA transcription.

Meyyappan M, Atadja PW, Riabowol KT (1996)



Since these are transcription factors and affect transcription of DNA, it becomes easy to see that a biological system might target these genes to control neurogenesis. My goal is to determine whether three of these genes (*snail*, *worniu*, and *cubitus interruptus*, Cai et al., 2001, Culi et al., 2006) are indeed regulatory targets of Pros and CycE and to further characterize the regulatory and functional interactions, and/or phenotypic consequences of these interactions, between these genes and Pros/CycE during Drosophila neurogenesis. By learning more about the function of these genes during the development of the central nervous system [CNS] and peripheral nervous system [PNS] of the model

system Drosophila, I can gain insight about how these genes function in humans and the possible regulatory effect of human Prospero (Prox1) and CycE in the regulation of cell differentiation and organogenesis.

The main question at the basis of my research addresses on how cell differentiation and proliferation is regulated during neurogenesis of Drosophila. While this question is very broad, my immediate research in the context of my honors thesis focused on whether the three Zn-finger transcription factor genes mentioned above are indeed regulatory targets of Pros and CycE.

Zn-finger transcription factor genes were determined as probable regulatory targets of Drosophila Pros and CycE, I performed a series of *in situ* hybridization experiments. This allowed me to localize and detect the specific transgenic mRNA sequences in the embryonic tissue of Drosophila embryos at different stages of their development. As the name suggests, this is done by hybridizing labeled complementary RNA to the sequence of interest. In our case, *in situ* hybridization reactions were performed over a three day period. A probe was generated for *in situ* hybridization, the DNA of the target gene will be amplified by a polymerase chain reaction (see figure 2) so that enough of the target DNA (modified to contain a T7 RNA polymerase recognition site) is acquired to make an antisense RNA probe. In making the probe, I used Digoxigenin labeled UTP which will allow me to tag and trace that specific RNA probe within the embryo and visually see it once the color reaction of the *in situ* hybridization reaction was performed.

Figure 3:

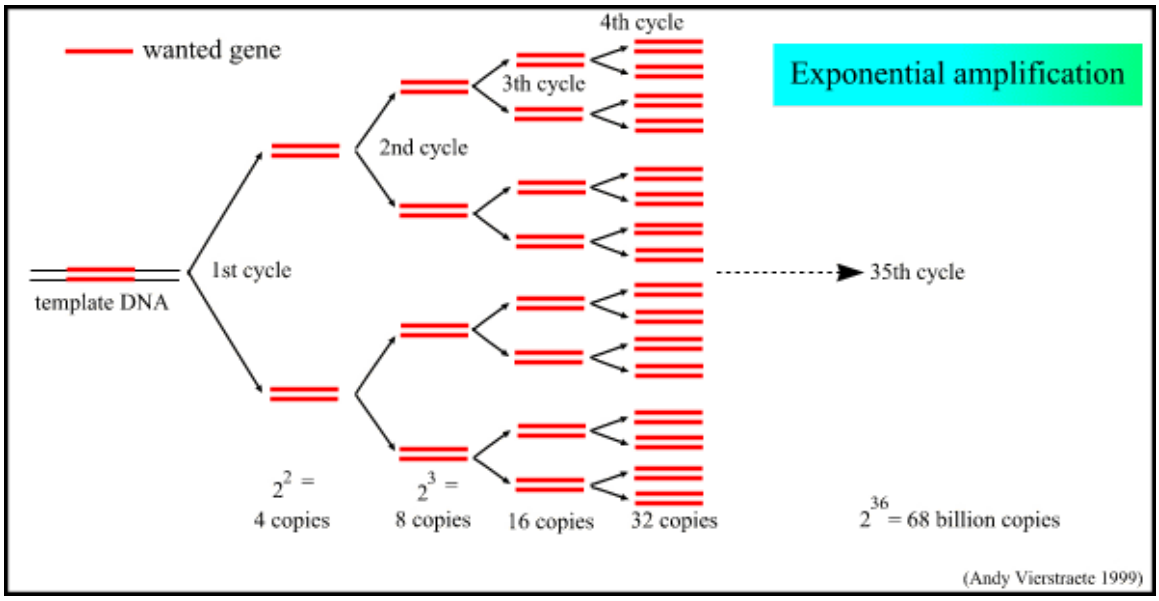
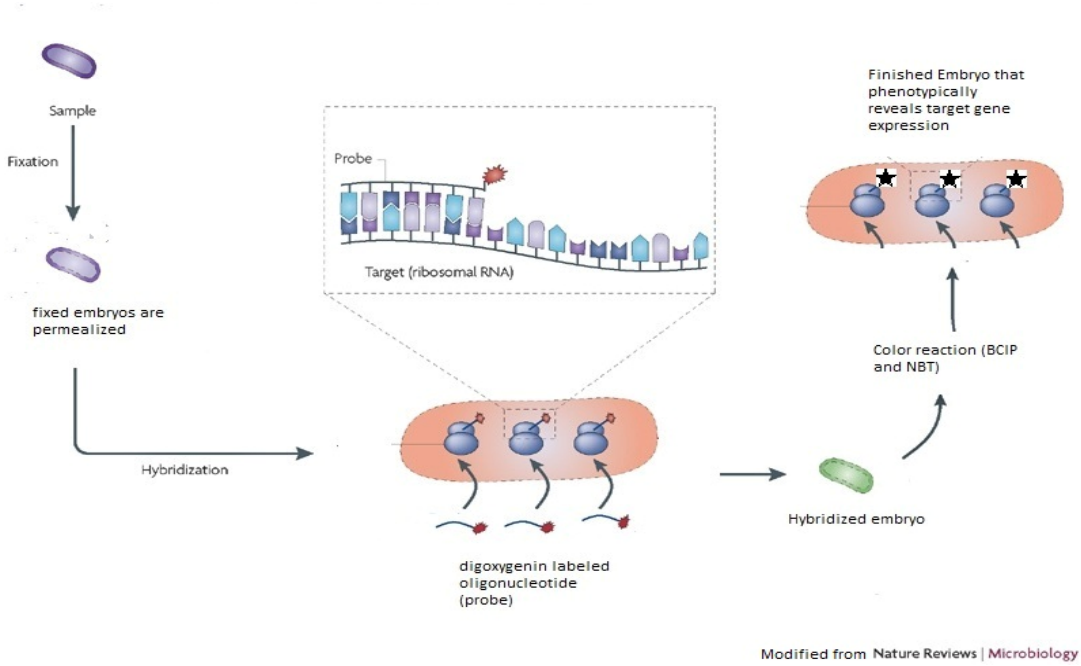


Figure 2. Diagram revealing how PCR can amplify a target gene exponentially. Cycling DNA in certain temperatures within an environment that promotes DNA replication (DNA Primers, dNTP mix, amplifications buffer, and genomic DNA). See Materials and Methods Section for a further description of PCR. (Andy Vierstraete, 1999)

Figure 4:



(modified from Rudolf Amann & Bernhard M. Fuchs, 2008)

However, just doing in-situ hybridizations of wild type Drosophila simply showed me where these genes end up being expressed normally and will not enable me to see how transcription factor genes affect neurogenesis and their potential as regulatory targets of Pros and CycE. To see if these genes do possess these qualities, I performed a series of tests. First, I compared the *in situ* hybridizations done with wild type Drosophila embryos with those of mutant Drosophila embryos where either Pros or CycE alone, or both together have been up regulated by ectopic expression to see how this affects the expression of these Zn-finger candidate genes. These experiments allowed me to determine whether these three candidate genes are indeed target genes of Pros and the coordinating function of Pros and CycE. To determine the biological relevance of the observed change in Zn-finger transcription factor gene expression, I set up crosses of Drosophila with mutant loss of function alleles of the Zn-finger transcription factors with Drosophila that are either wild type, or carry mutant alleles of Pros, CycE, or both Pros and CycE. Immunohistochemistry with various antibodies that allow the visualization of CNS/PNS, mitotic activity, and neuronal differentiation steps, was used to analyze the embryos recovered from these crosses. Microscopy was used to image the antibody labeled embryos.

In summary, I attempted in the following to show the expression of a set of three candidate Zn-finger transcription factors in various genetic backgrounds to determine whether they are regulating targets of Pros and CycE. While, due to technical problems and time limitations, this could not be accomplished for all three genes, I will show a full set of data for one gene (*worniu*) that indicate that it may indeed be a regulatory target of Prospero and Cyclin E. In addition, I will present additional, incomplete data sets for the

other two genes (*cubitus interruptus* and *snail*).

MATERIALS AND METHODS

***Protocols and procedures follow closely with the Vaessin laboratory manual/protocol and Li and Vaessin, 2000**

2.1 Embryo Collection

2.1.1 *Drosophila* Cyclin E (p[p^2]cycE/CyO), Prospero ($prosJ^{013}$/TM3,Sb)and Prospero/Cyclin E (CycEAR95/CyO, act lacZ ; $prosJ^{013}$/TM^3,Sb, act lacZ) mutants designated as cycE, pros, and cycE/pros mutants were prepared for embryo collection.

i Expand fly stocks to get respectable numbers for embryo collection. Getting larger collections will actually save time as fewer collections would be needed to obtain the same amount of embryos

ii Seemed to work best if the container used were medium to large size (approx. 1L). Bottoms of containers should be cut off and replaced with mesh fabric attached with Gorilla Glue providing the flies with plenty of oxygen. Petri dishes can be used as grape plates for the tops of the containers (see grape plate preparation at end of 2.1.1). Place a quarter-size amount of yeast on the grape plate. Once all preparations are complete, flies of the same type can be placed in the container using a funnel and the grape plate can be used to cover the top. Tape the grape plates on securely so no flies can escape and flip the container so the grape plate is on the bottom. Leave at room temperature

2.1.2 Collection of Embryos (making sure each mutant remains isolated from the other).

i Wash embryos into a collection basket with water using a Q-tip and rinse until no traces of yeast are visible.

2.1.3 Dechorionate embryos

i Place collection basket containing the embryos in a Petri dish containing 50% commercial bleach (with water) and on the shaker for approximately 5 minutes. The chorion of the embryo has been removed when the surface of the embryo appears shiny. This is due to the vitelline membrane becoming visible. It is usually better to go a little too long in the bleach then to risk the embryos still having their chorion attached.

ii Rinse in water until no bleach is present.

iii Transfer embryos to a vial with a lid containing the fixation solution.

2.1.4 Fixation of Embryos to stabilize embryonic tissue

i Place vials containing appropriate embryos and solutions on shaker at 300 rpm for 20 minutes (tape down to secure them).

ii Discard lower aqueous phase [formaldehyde waste] as completely as possible as this phase will interfere with the devitillinization of the embryos.

2.1.5 Devitillinize embryos

i Add at least an equal amount of methanol to the upper phase which contains the embryos.

ii Shake vigorously for one minute [The devitillinized embryos will sink to the bottom. Shake again if a high percentage still have their membrane]

iii Discard the upper phase [heptanes waste], inter-phase [including embryos] as well as most of the lower phase

2.1.6 Dehydration of embryos for storage

i Add 5 ml of methanol to the vials containing the embryos.

ii Exchange with 5 ml Methanol

iii Move embryos in 1.7 ml micro centrifuge tube

iv Wash 4x with 1ml 100% ethanol

v Store at -20^{o}C in 100% ethanol

2.2. Making the dig trace probe for each zinc-finger transcription factor gene (*snail, worniu, and cubitus interruptus)*

2.2.1 PCR Reaction: Amplification of Target DNA

i 3' primer contained the T7 RNA polymerase binding site

ii Place following contents into a micro eppendorf tube and placed into thermocycler

For 100 ul reaction

- 50 ul PCR mix 2x
- 5 ul forward primer
- 5 ul reverse primer
- 5 ul genomic DNA
- 35 ul DDH_2O (primers designated to each segment of interest)

iii thermocycler settings

- 94^{o}C 10 minutes (initial, only needs to be done once)

- Repeat following steps 25 times
- 94^{o}C for 30 seconds
- 55^{o}C for one minute
- 72^{o}C for one minute

2.2.2 Making the dig trace probe

i Dig labeled RNA was generated using the MegaScript kit

ii Place the following contents into a micro eppendorf tube (separately for each gene) and placed overnight at 37^{o}C

- Total rxn volume = 10 ul
- 4 ul pcr product
- 0.75 ul ATP
- 0.75 ul CTP
- 0.75 ul GTP
- 0.75 ul UTP
- 1.3 ul digoxygenin UTP (Roche)
- 1 ul 10x reaction buffer
- 1 ul enzyme mix

2.2.3 Checking the PCR product Dig-trace probe via gel electrophoresis

i Make the gel by first adding 50 ml of TAE Buffer into an Erlenmeyer flask

ii Add 0.5 Agerose powder (to give 1% composition

iii Heat in microwave until close to a boil and stir to dissolve the agarose completely

iv Add 1 ul ethidium bromide and stir

v Pour gel into gel apparatus

vi Load each chamber

- 1st Chamber: 2 ul DNA Ladder

- 2nd Chamber: 5ul PCR Product, 13 ul ddH_20, 2 ul Loading Buffer

- 3rd Chamber: 0.2 ul Probe, 9.8 ul ddH_20, 2 ul Loading Buffer

vii Make sure PCR product and probe sizes are consistent with their expected sizes

2.3 *in situ* Hybrization

2.3.1 General description of *in situ* reactions

i First the embryos must be treated to allow permeabilization which allows the probe to reach its target mRNA sequence. These RNA sequences are often protected by proteins and even the act of fixation results in the cross-linking of proteins. To permeabilize the embryos I will treat them with a small amount of Protease K. Protease K is an endopeptidase that will destroy all peptide bonds within a broad pH so you have to be precise on the amount used and the time it is used. If too little is used or for a short duration of time, the protein coatings will still be intact and the probe will not be able to reach its target, but if too much is used or for a long duration of time then it will destroy the organism and its cell integrity. A post fixation step is then needed to help stabilize the tissue. Next, the embryos must be treated with the same solution as in the hybridization step to reduce background staining. The actual hybridization includes the probe and allows the probe to anneal to its complimentary mRNA strand over night. The next day is unbound probe is washed away. Finally, after dehydrating the embryos they can be mounted and examined under a microscope. When everything runs correctly, I am able to detect and localize where a gene is expressed and how it is expressed at the different stages of embryonic

development. Comparative studies between the cycE (wild type), Pros, and cycE/Pros mutants must be done at the same developmental stages to see how the expression is changed to determine the regulatory effects of each protein on the target zinc-finger transcription factor gene. The staging is according *The Embryonic Development of Drosophila melanogaster*, J.A. Campos-Ortéga, V Hartenstein (1985) The stages examined most from my experiment were stages 13-14.

2.3.2 Outline of in-situ protocol

2.3.3 Rehydration of embryos after storage (after each exchange place on nutator for 2 minutes)

i Place embryos into a 1.7 ml eppendorf tube

ii Exchange solution containing the embryos with 1 ml 25% Na-PBT / 75% Ethanol

iii Exchange solution with 1 ml 50% Na-PBT / 50% Ethanol

iv Exchange solution with 1 ml 75% Na-PBT / 25% Ethanol

v Exchange solution twice with 1 ml 100% Na-PBT

2.3.4 Fixation of embryos in Na-PBT / 5% Formaldehyde (344 ul Na-PBT w/ 156 ul of 16% formaldehyde)

i Incubate embryos in 500ul fixation solution for 25 min at RT with agitation [on Nutator].

ii Wash 4x: each: 1ml Na-PBTw for 2 min at RT with agitation [Nutator].

2.3.5 Protease K treatment:

i Exchange to 500ul Na-PBTween20 and add x ul Protease K stock solution on ice.

ii Incubate embryos in this solution for 5 min at 25 ^{o}C on Nutator [in 25 ^{o}C incubator].

iii Add 1ml Na-PBTw

iv Rinse 4x with 1ml Na-PBTw each [2x on ice/ 2x at RT] {to remove Proteinase K activity}.

2.3.6 Post fixation in Na-PBTw/ 5% Formaldehyde: [344ul Na-PBT /156ul 16% Formaldehyde]

i Incubate embryos in 500ul fixation solution for 25 min at RT with agitation [on Nutator].

ii Wash 4x with 1ml Na-PBTw for 2 min each at RT with agitation [Nutator]

2.3.7 Pre-hybridisation:

i wash 2x with 500ul Hybridization buffer, RT, agitation [Nutator], 5 min each

ii Exchange buffer for 500ul 100% hybridization buffer and incubate at 55oC, 300rpm [Eppendorf] for >1 hour to ON.

2.3.8 Hybridisation:

i Dilute probe [1ul probe in 25 ul Hyb solution]

ii Denature for 3 min at 95 ^{o}C, cool for 3 min on ice, centrifuge shortly.

iii exchange pre-hybridization buffers against probe in hybridization buffer [25ul] and incubate at 55^{o}C, 300rpm [Eppendorf] ON [12-18 hours]. (Store used probe at –80oC for reuse.)

DAY 2:

2.3.9 Preabsorbtion of anti-Dig antibody [in parallel to B]:

i Rehydrate 100 ul Embryo: wash 3-4x with 1ml Na-PBT for 5 min each at RT

ii Incubate for 30 min in 1ml Na-PBTw-1%BSA at RT, Nutator

iii Dilute Antibody [Roche: anti-Digoxigenin-fab-fragment-AP-conjugated] to 1:500 in Na-PBT-1%BSA and incubate for 1 hour with embryos at RT

iv store at 4oC till use.

2.3.10 Washes of hybridized embryos: [Nutator]

i 4x for 20- 30 min each in 500ul Hyb buffer at 55oC

ii 1x for 15-20 min in 500ul 50% Hyb buffer/ 50% Na-PBTw at RT, agitation [Nutator]

iii 1x for 15-20 min in 500ul 25% Hyb-buffer/75% Na-PBTw at RT, agitation [Nutator]

iv 5x for 7-10 min each in 1ml Na-PBTw at RT

2.3.11 Antibody incubation:

i Add preabsorbed Antibody [anti-Dig0xigenin-Fab-fragment AP-conjugated] at 1:4000 final dilutions in Na-PBTw-1%BSA: [62.5ul preabsorbed AB in 437.5ul Na-PBT-1%BSA] and incubate at 4^{o}C for ON [or 2 hours at RT] on Nutator

DAY 3:

2.3.12 Washes [Nutator]:

i 4x for 10- 15 min each in 1ml Na-PBTw at RT

i 2x for 2-5 min each in 500ul AP-buffer at RT

2.3.13 Color reaction:

i Remove last wash and add 493 ul AP-buffer plus 5 ul NBT [50mg/ml, Promega] and 1.875 ul BCIP [50mg/ml, Promega]

ii Place about 250ul of the embryos in viewing dish and check color development in regular intervals [keep in dark in between]

iii Stop color reaction with at least equal volume 70% Ethanol

2.3.14 Dehydration of larvae: exchange solution after 2 min on Nutator

i 1x: 1ml 50% Ethanol / 50% Na-PBTw

ii 1x: 1ml 75% Ethanol / 25% Na-PBTw

iii 2x: 1ml 100 % Ethanol

iv 2x: 1ml fresh 100% Ethanol

v 800ul Xylenes [make sure xylenes does not turn cloudy, if: repeat 2 more 100% Ethanol washes and exchange against xylenes again] [if Xylenes gives you a headache, you can try toluene instead]

vi store in xylenes at 4^{o}C or RT

2.3.15 Mounting:

i Transfer embryos in small amount of xylenes on microscope slide

ii Dry xylenes off with Kim wipes

iii Add 4-5 drops of Paramount and mix well with embryos [needle].

iv Cover with 50mm cover slide and store on slide warmer for at least ON [best 24-48 hours]

v look at under the microscope to see if embryos have been labeled

vi Store horizontal for several weeks as mounting medium stays soft for a long time

2.4 Microscopy

2.4.1 Taking pictures of embryos under microscope to be able to compare them

i Take multiple images of labeled embryos attempting to capture embryos that are in similar developmental stages

ii Compare the images from wild type (cycE), Pros, and Pros/cycE for each probe to determine if that gene is a regulatory target of Pros or cycE.

2.5 Common Solutions Needed

2.5.1 10x Na-PBS [old Rubin/Vaessin lab recipe]:

For 1L of solution:

i 75.97g 1.3M NaCl
ii 9.937g 70 mM Na_2HPO_4 anhydrous
iii 3.599g 30 mMNaH_2PO_4 anhydrous
iv PH: 6.8 [shifts to pH7.4 when diluted to1x]

2.5.2 Na-PBT: 1xPBS plus 0.1% Tween-20

i IS: Tween-20 0.1% [Na-PBTw]

2.5.3 *in situ* Fixation solution: 50% Heptanes/10% Formaldehyde in 1x PBS

For 10 ml

i 5 ml Heptanes
ii 3.15 ml H_2O
iii 1.35 ml 37% Formaldehyde
iv 0.50 ml 10x PBS

2.5.4 Fixation solutions: Na-PBT with 5% Formaldehyde

For 500 ul

i 344 ul 1x Na-PBT
ii 156 ul 16% Formaldehyde [with out Methanol]

2.5.5 Protease K: [Roche: Proteinase K, recombinant PCR grade 100mg #03 115 879 001]

i 10mg/ml or 20mg/ml stock solution in 10 mM Tris ph7.5 or Na-PBS, aliquot, flash frozen in liquid N_2 and stored at -80^{o}C.

ii Use each aliquot only once as repeated freeze/ thaw cycles decrease activity of the enzyme.

2.5.5 Pre-/ Hybridization-buffer:

For 100 ml:

i 50 ml 50% [deionizer] formamide
ii 25 ml [20x]5x SSC
iii 100 ul O.1% Tween-20
iv 50 ul 50ug/ ml Heparin [100mg/ ml]
v 500 ul [10 ug/ ml tRNA [20mg/ ml]
vi H_20 to 100ml

2.5.6 AP-Buffer: [Roche: Jan 2008] 50 ml

i 5 ml [1M]100 mM Tris HCL ph: 9.5
ii 1 ml [5M]100 mM NaCl
iii H_20 to 100ml

RESULTS

3.1 Analysis of ectopic over expression of *worniu*

Phenotypical analysis of *worniu* mutant embryos was performed by in situ hybridization with the over expression of Pros and Pros/cycE using the KrGal4 system. The part of the experiment was carried out together with Michael McKee, a former undergraduate in the Vaessin Laboratory. This allowed for the phenotype of mutant embryos to be studied to test whether *worniu* is a regulating target of Pros, cycE, or both. To do so I compared the wild type embryos that were labeled using a probe for *worniu*. One can visually see the labeled cells under a microscope as those cells will have a dark-bluish tint where the gene is transcribed and expressed. This is due to the fact that antibodies (anti-dig) that attach to the digoxygenin probe that was created can undergo a color reaction using NBT and BCIP. In figure 5A, the places within the embryo that are over expressed by the Kruppel-Gal4 system is the last thoracic and first few abdominal segments as indicated by the arrows. These are the areas that are compared to analyze *worniu* expression.

3.1.1 Analysis of *worniu* with UAS-cycE ectopic over expression

It appears CyclinE barely effects worniu expression or not at all. This observation would be in line with the previous observations based on microarray analysis in the Vaessin laboratory.

3.1.2 Analysis of *worniu* with UAS-ProsK ectopic over expression

It appears Prospero suppresses *worniu* expression. Figure 5B reveals this as it is much lighter (less labeled) than the cycE embryo 5A or wild type (not shown).

Also, one can compare the expression in the last thoracic and first few abdominal segments to the rest of the embryo to see that it is also under expressed in this area which is not seen in figure 5A.

3.1.3 Analysis of *worniu* with UAS-ProsK/UAS-cycE ectopic over expression

It appears that Prospero and Cyclin E expressed together cause worniu expression to be more similar to that of cycE alone (figure 5A) or wild type (not shown).

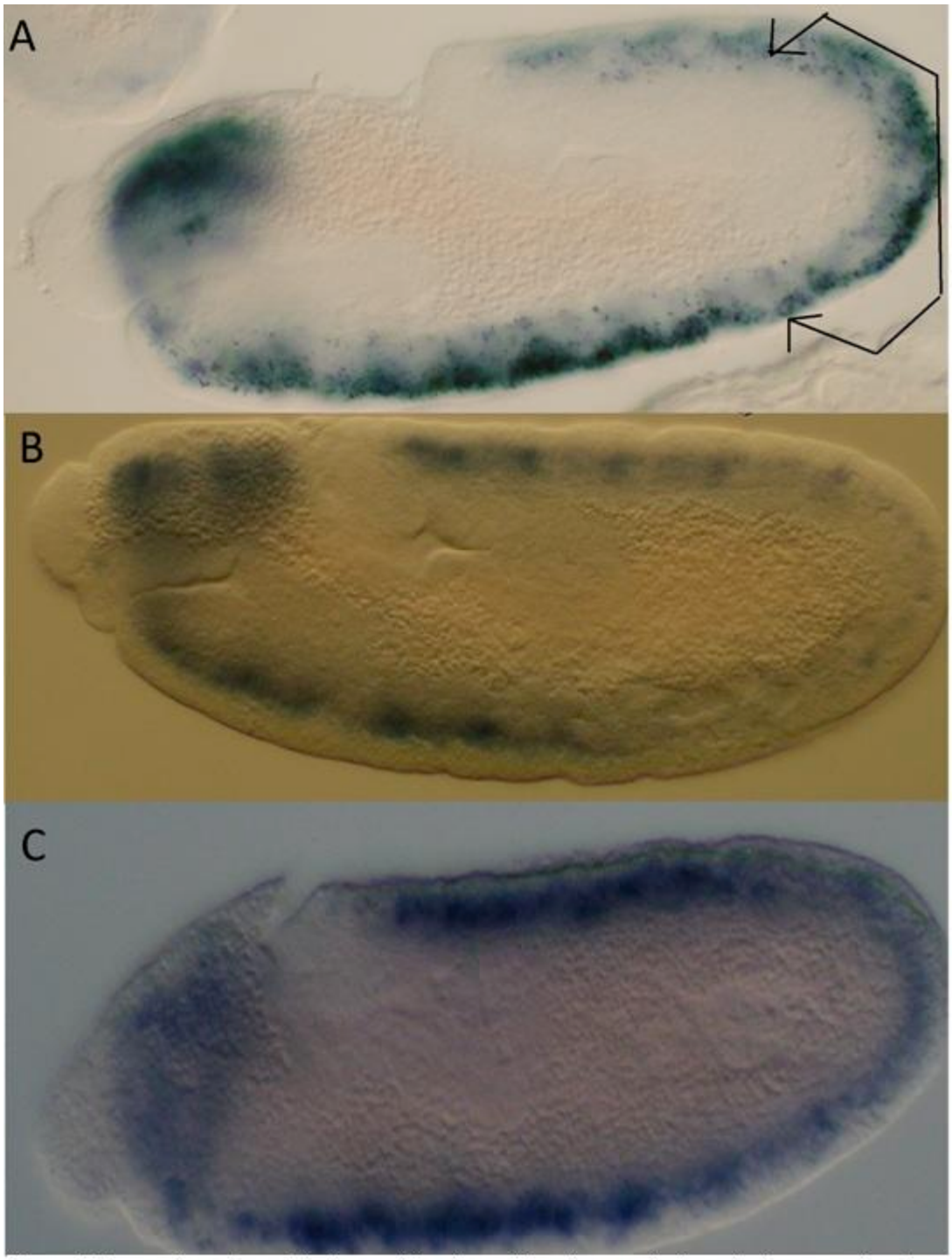


Figure 5: Expression of worniu in Drosophila stage ~12 embryos with the ectopic expression of cycE (A), Pros (B) or cycE and Pros together (C). Lines in A indicate area of overexpression. All embryos are shown at the same magnification.

3.2 Analysis of *worniu* with mutant embryos (loss of function in cycE, Pros, and both)

Phenotypical analysis of *worniu* in *cycE, pros*, and cy*cE/pros* mutant embryos was performed by *in situ* hybrization experiment.

3.2.1 Analysis of *worniu* with p[p^2]cycE/CyO (cyclinE mutant)

It appears loss of *cyclinE* barely effects worniu expression or not at all. This observation would be in line with the previous observations based on microarray analysis in the Vaessin laboratory. Figure 6A shows expression as expected in a wild-type embryo.

3.2.2 Analysis of *worniu* with pros J^{013}/TM3, Sb (Prospero mutant)

It appears loss of *prospero* upregulates *worniu* expression. Figure 6B reveals that the *worniu* gene is more expressed in the Prospero mutant as the labeling is both thicker and darker (meaning more cells have been marked).

3.2.3 Analysis of *worniu* with CycEAR95/Cyo,act-lacZ ; J^{013}/TM3,Sb,act-lacZ (both CycE and pros mutant)

It appears that the Prospero and Cyclin E mutants together causes *worniu* expression to revert back to a near-wild type levels as seen by the similarities between Figure 6A and figure 6C.

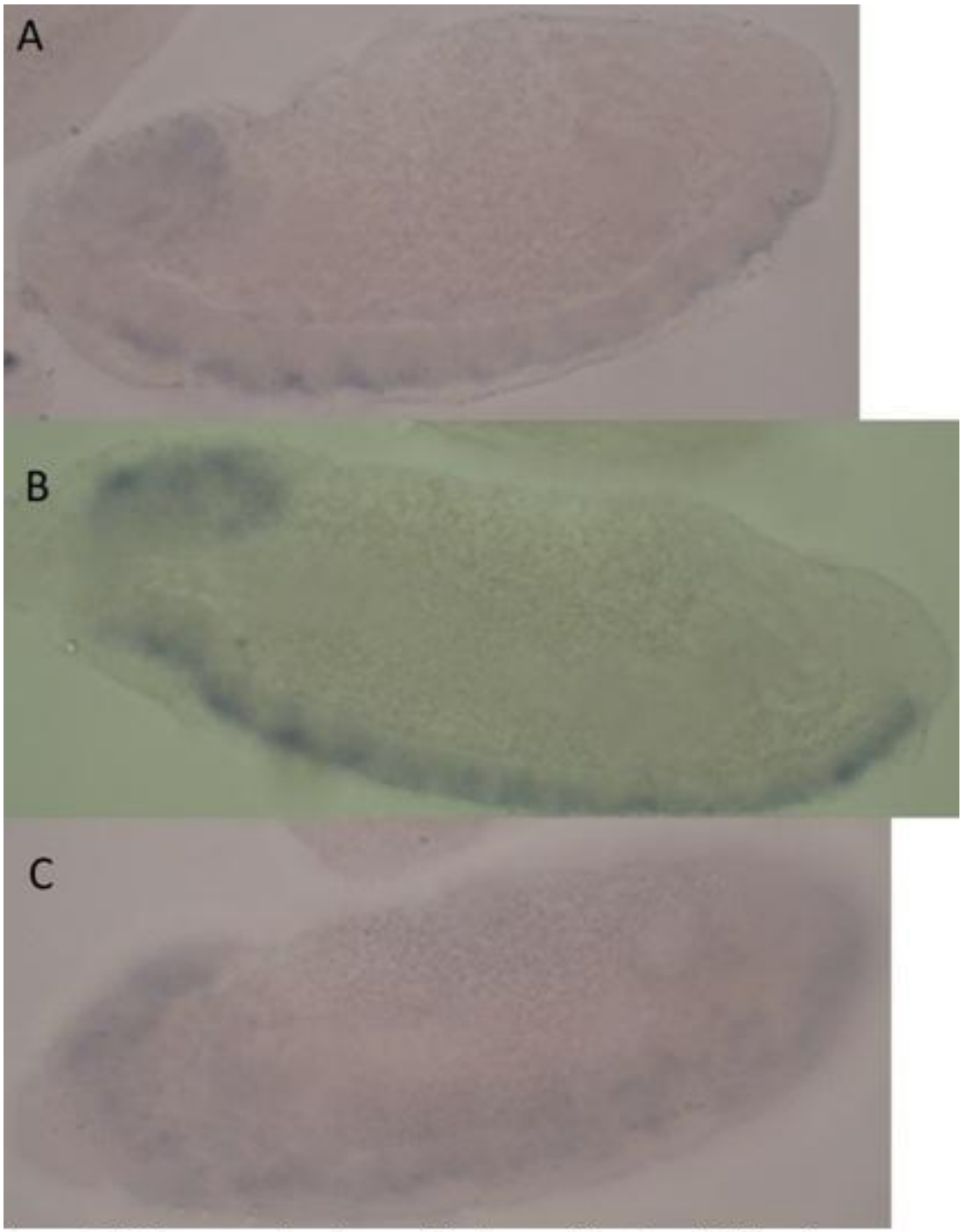


Figure 6: RNA expression of worniu in Drosophila stage 13/14 embryos

A: p[p2]cycE/CyO: worniu expression is equivalent to wild type (not shown)
B: ProsJ013/TM3,SB: worniu expression is higher than in control
C: CycEAR95/Cyo,act-lacZ; prosJ013/TM3,Sb,act-lacZ : mutant resembles wild type. All embryos are shown at the same magnification

3.3 Analysis of *cubitus interruptus*

3.3.1 Expression of *cubitus interruptus* in embryos with ectopic expression of cycE, Pros, cycE/Pros behaved similar to worniu (personal communication, McKee and Vaessin)

3.3.2 Analysis of *cubitus interruptus* expression.

It appears that loss of cyclinE barely effects *cubitus interruptus* expression (fig 7A), as compared to wild type (not shown). This observation would be in line with the previous observations based on microarray analysis in the Vaessin laboratory (Vaessin, personal communication).

3.3.2 Analysis of *cubitus interruptus* with $pros^{J013}$/TM3, Sb (Prospero mutant)

There may be a slight reduction of *cubitus interruptus* expression (7b) detectable.

3.3.3 Analysis of *cubitus interruptus* with CycEAR95/Cyo,actB ; $pros^{J013}$/TM3,Sb,actB (both cycE and pros mutant).

Different to worniu, I did not see a distinct difference in expression in *ci* in either cycE or Pros, however, in the double mutant there seems to be a shift of expression into the nervous system while in just the Pros or cycE most of the expression is predominantly in the epidermis at these stages. The significance of this shift of expression into the nervous system in the double mutant. of this is presently not clear.

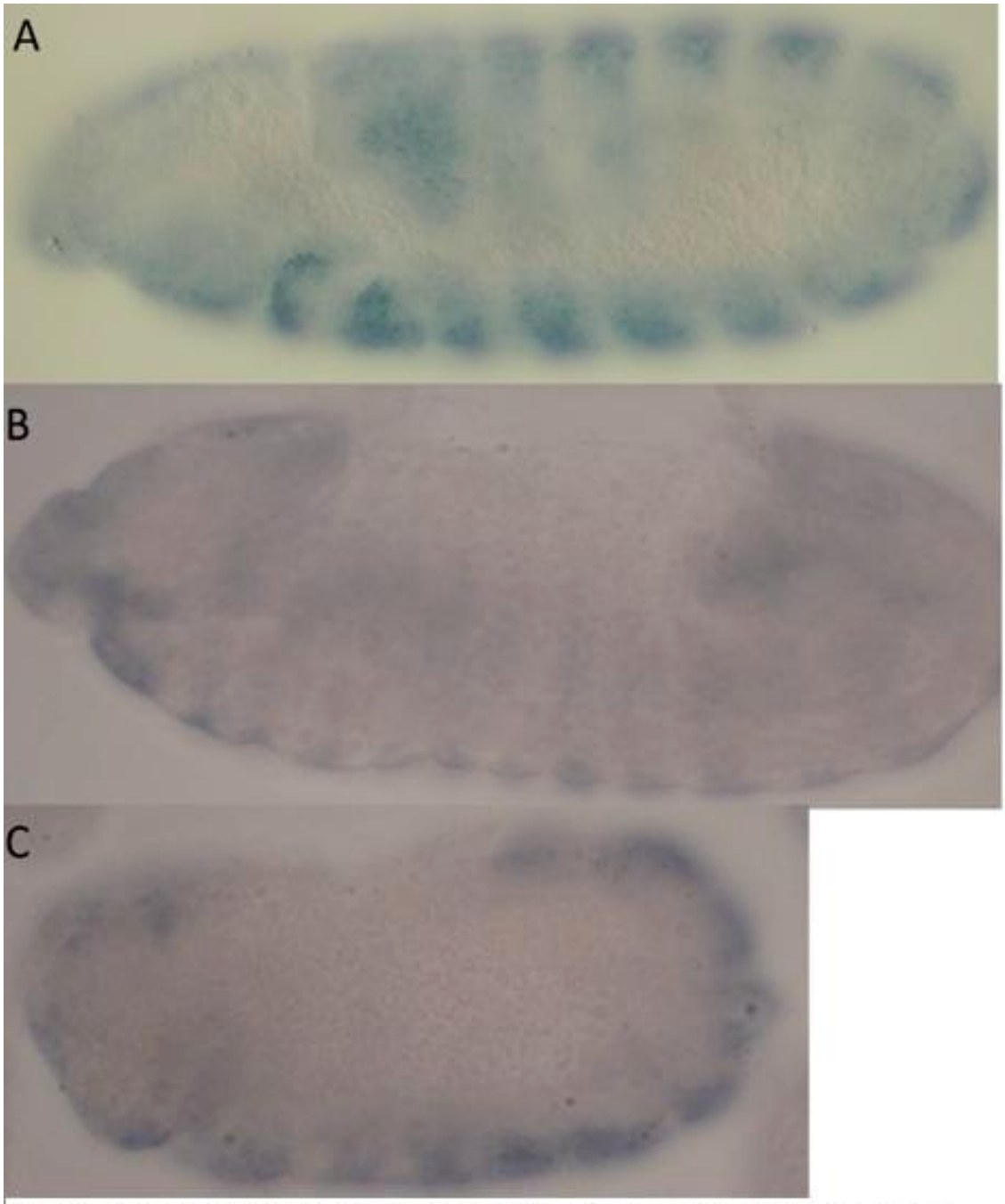


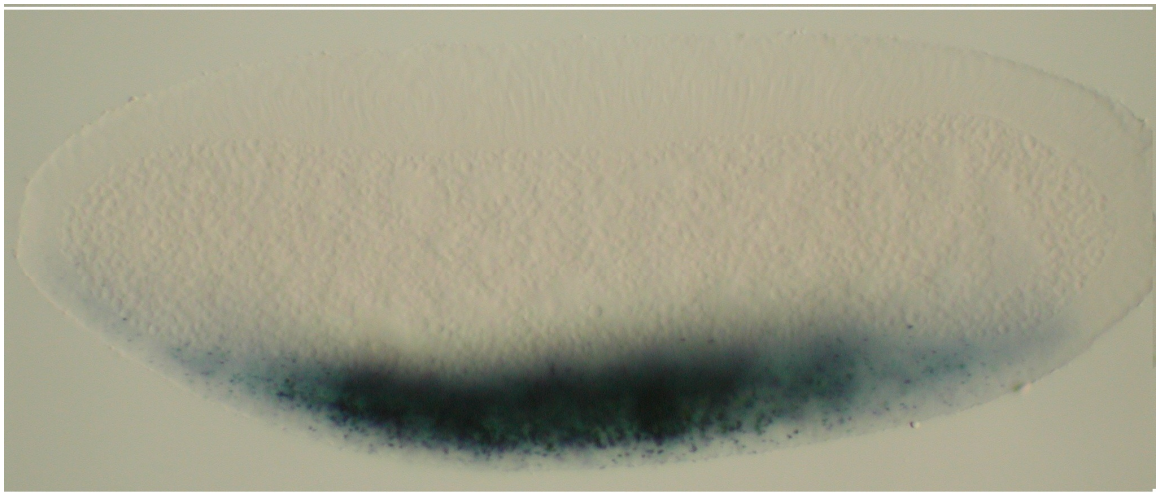
Figure 7: RNA expression of cubitus interruptus in Drosophila stage 12 embryos

A: p[p2]cycE/CyO: cubitus interruptus expression is equivalent to wild type (not shown)
B: ProsJ013/TM3,SB: cubitus interruptus expression is slightly lower than control
C: CycEAR95/Cyo,act-lacZ; prosJ013/TM3,Sb,act-lacZ : A shift in expression can be observed

3.4 Analysis of *snail*

3.4.1 Even though the snail probe was tested with gel electrophoresis and *in situ* hybridization (figure 8) and seemed to work fine in both cases, the probe did not seem to label any of the genes in subsequent reactions. Presently the reasons for this is unknown but the most likely explanation to this phenomenon is that the probe was contaminated.

Figure: 8



Snail Expression in wild type embryo.

stage 4 embryo

DISCUSSION

4.1 General discussion about embryo collections.

Embryo collections seemed to work if they were done under certain parameters. Of course there may be bias to who is performing the experiment but these are the conditions that worked best for my experiment and me. I used cylinder containers that were approximately 1 L in volume that had fine netting on one side and would have the grape plate on the other side. For the grape plates I would use Petri dishes. The large size containers would allow me to place all flies within one container save a good amount of time during the collection as I would only be concerned with the embryos on one grape plate per target gene. As fly activity and growth changes due to temperature, the best temperature for me for the collections was at approx. room temperature (22-24^{o}C). This made it so the collection of the embryos would have to occur at 24 hours after a new grape plate was put on the container. This becomes significant because if you wait too long the embryos become larvae and the collection will be ruined. Also, I seemed to have less problems with larvae contaminations at 22-24^{o}C than doing collections every 44-48 hours at 18^{o}C. One should also wipe the tops of the containers with a Kim wipe before placing a new grape plate on as this can help get rid of larvae (once again easier with a large container instead of multiple small containers). Finally, it is better to place the embryos in bleach for a longer than needed time than too short to make sure they are all dechorionated (5 min).

4.2 General discussion about making the probes for each target gene.

While making a dig-trace probe is relatively simple and not very time consuming, it is one of the most important steps in an experiment such as mine. This becomes exemplified as one observes my experiments involving *snail*. When making the probe sterility becomes necessity as contamination by RNAse (which is abundant on many things especially our skin) will destroy the probe. Once made, the probe can then be tested by gel electrophoresis.

Figure 9:

Lane 1: 5 ul Gene Ruler DNA Ladder
Lane 2: 2 ul DNA Ladder (Hyperladder IV), 5ul H20
Lane 3: 2 ul Gene Ruler DNA Ladder
Lane 4: 2 ul PCR Product, 8 ul H2O, 2 ul LB
Lane 5: 0.2 ul probe (31813), 9.8 ul H20, 2 ul LB
Lane 6: 1 ul PCR Product (31813), 9 ul H20, 2 ul LB
Lane 7: 0.2 ul probe (Snail), 9.8 ul H20, 2 ul LB
Lane 8: 1 ul PCR product (Snail), 9 ul H20, 2 ul LE
Lane 9: 5 ul PCR product (Snail), 5 ul H20, 2 ul LE
Lane 10: 4 ul DNA Ladder (Hyperladder II)
LB = Loading Buffer
Gel runs from right to left

This data reveals that both the PCR product and probe has probably been correctly made as both are near the size they should be for that. This was from an earlier creation of *snail* and it was redone later at the same time that I made the *worniu* and *cubitus interruptus* probe. It also shows that when compared with the control, *31813*, it is not that concentrated as it gives much less of a signal. The best test is to run an *in situ* with wild type embryos and see if it matched with the data from flybase.org, which all of my probes did.

4.3 Failure of the *snail* probe

Since all steps with each of the three probes (and in one case a control with a 31813 probe and wild type) were done in unison with one another and the only variable was the probe, then the failure of the labeling of *snail* expression had to lie in a problem with the probe) Even though I tested the quality of the probe I made both by gel electrophoresis and an *in situ* reaction with wild type embryos and they both worked fine, the probe did not work in the following experiments with the mutant embryos. This brings me to a conclusion that the problem is most likely that the *snail* probe at some point became contaminated. It is most probably that RNAse destroyed the probe as it revealed no signal at all.

4.4 *worniu* may be a regulatory target of both CyclinE and/or Prospero

The zinc-transcription factor gene, *worniu*, appears to be a regulatory target of CyclinE and Prospero as one compares the products of the *in situ* hybridizations performed. The comparison between wild type embryos and embryos with mutations within the *prospero* and *cyclinE* genes strengthen the argument that *worniu* might be a regulatory target of those proteins in CNS development. The expression or worniu in an ectopic over expressed *prospero* mutant was lower than in that of the ectopic over expression in wild type, see figure 5. CyclinE also seems to have a regulatory effect. While one might not be able to see it when comparing just the cycE over expression mutant with that of wild type as they are so similar, comparing the cycE, Pros, and cycE/Pros embryos reveal a regulatory effect. Pros over expression caused lower expression but the addition of overexpression of cycE mutant causes the expression to be upregulated back close to that in wild type. Perhaps the cyclinE modifies Pros activity so that it masks its

effects in the double over expression embryos. These results run parallel with that of the microarrays done previously in the Vaessin laboratory. This result becomes even more reputable as the ectopic (under) expression of *worniu* in the three mutants has corresponding results, see figure 6. The Pros mutant now expresses *worniu* more than the cycE or cycE/Pros whose expression is both similar to that of wild-type.

4.5 *cubitus interruptus* may also be a regulatory target of both CyclinE and/or Prospero

The zinc-transcription factor gene, *cubitus interruptus,* appears to be a regulatory target of CyclinE and Prospero as one compares the products of the *in situ* hybridizations performed. The analysis is done in the exact same way as for *worniu* described previously by comparing *in situ* hybridizations of ectopically expressed genes as well as loss of function mutants. The *prospero* and *cyclinE* genes reveal that *cubitus interruptus* might indeed be a regulatory target of those proteins in CNS development. The main evidence for this is the shift in expression from the epidermis to the CNS. Much analysis of cubitus interruptus is need as there are too many discrepancies that must be accounted for. Figure 7A and 7B are difficult to compare as figure 7A is in an earlier developmental stage. Also as it is not sure whether the shortened embryo is caused due to the double mutant mutant, a dual marking experiment, as described in the next section, is needed to make sure that 7C is the dual mutant. However, if these results are as they were interpreted then they run parallel with that of the microarrays done previously in the Vaessin laboratory.

4.6 Further investigation of the zinc-finger transcription factor genes as regulatory targets of cycE, Pros, or both.

i A better way to be able to distinguish the differences between the genotypes than just observing the phenotypes would be to use a double label experiment where you use genetic markers that labels an easily distinguishable genetic marker on balancer chromosomes (such as lacZ). Because of this, one can distinguish the double mutant from any other genotype as all others will either be labeled for the lac-Z gene or appear to be wild-type.

ii Assuming a embryo with a marked genotype validates the observation, genetic interaction studies could be performed to further investigate the regulation of zinc-finger transcription factors by Prospero and CyclinE.

iii As in all research, the results that are to be presented must be supported by a substantial amount of experimental data. All of these experiments must be repeated several times to make sure that the results are both consistent and repeatable.

4.7 Neurogenesis and its importance

i When dealing with complex developmental processes such as neurogenesis, it is quite easy to see how minor changes in just one gene can have drastic effects. During neurogenesis, new neurons and gill cells are being formed. This occurs in Drosophila initially during embryonic development and is repeated later again during larval/pupil development. Neurons transmit information by electrochemical signaling and allow the body to react to stimuli, grow, and even learn. The importance of CNS and

axon guidance is prominently displayed in the effects of neurodegenerative diseases, such as Parkinson's or Huntington's disease that put strain on the CNS. In Huntington's disease, a single gene is the cause of many physical and psychological consequences that shows just how serious neuronal decay is and how one gene can greatly affect a whole organism. I can also see the importance of understanding the regulation of cell division and cell cycle regulators during neurogenesis as I look at diseases such as brain cancer (Ashraf et al., 2004; Caussinus and Hirth, 2007). In cancer, the regulation of cell division is amiss and this in turn can cause tumors. Learning exactly how these processes work and how they can go awry may help to provide future avenues to develop cures and the development of new treatment approaches. Furthermore, with enough knowledge of the regulatory interplay of mitotic activity and neuronal differentiation, I may be able to manipulate cell mitotic division and differentiation to heal a person who has had a spinal or brain trauma. As with many applications in molecular biology, when people understand more on basic model systems, they will be able to use this data to more accurately determine the way more complex organisms develop and function.

4.8 Importance of research with zinc-finger transcription factor genes

i Because many biological pathways are similar between organisms, learning more about a simpler model system will give insight to more complex organisms. Development and regulation of the central nervous system is very complex even in the simplest of organisms, but research such as my project must be done to gain greater knowledge of it. Research like this could lead to medical treatments later. It may very well be a gateway to a greater understanding of diseases such as brain cancer. Progressive neuronal loss characterizes these disorders, but much research has been done

showing that neurogenesis may continue in adults and could combat these disorders. Neurogenesis has so many applications that it has been shown to increase memory if it is up-regulated, or cause other disorders such as depression if down-regulated. This shows how neurogenesis has such a broad spectrum of purposes within an organism. Learning about such a vital process as neurogenesis facilitates subsequent research. While this research may just serve to identify and further characterize transcription factor genes as regulatory targets of Pros/CycE during Drosophila neurogenesis, some day research could stem from this that has a significant effect on the lives of many individuals.

CONCLUSION

In summery, as indicated by microarray analysis in the Vaessin Laboratory it does seem that the zinc-finger transcription factor genes, *worniu* and *cubitus interruptus*, are regulatory targets of the proteins Prospero and CyclinE. *in situ* hybridizations indicate that Prospero and CyclinE regulate gene expression of these transcription factors. The proteins Prospero and CyclinE had varying effects. CyclinE had little effect on its own, Prospero either promoted or suppressed expression, and the double mutant cycE/Pros had similar expression to that of wild type embryos or would cause a shift in where the expression takes place (*cubitus interruptus*).

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